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Characterizing Importin Binding to Thyroid Hormone Receptor α1

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Characterizing Importin Binding to Thyroid Hormone Receptor α1

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by

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Abstract

Thyroid hormone, or T_3, is essential in many bodily functions, from early development to the maintenance of health in adults. It is crucial for growth and skeletal development, development of the nervous system, cell differentiation, and maintenance of metabolic balance. The thyroid hormone receptor, TR, is a major mediator of thyroid hormone action. TR is a transcription factor and able to activate or repress transcription depending on the binding of its ligand, T_3. There are two isoforms of TR, encoded by different genes: TRα and TRβ. Each of these isoforms have multiple alternative splicing products.

While TR's main function is carried out in the nucleus, multiple studies have shown that TR is shuttled rapidly between the nucleus and cytosol. Mislocalization of TR can be linked to diseases such as T_3 resistance and cancer. Nuclear localization is mediated by importins, which bind to TRα by recognizing nuclear localization signals (NLSs).

Previous studies have shown the presence of two NLS in the TRα1 isoform: in the Hinge domain (NLS 1) and in the A/B transactivation domain (NLS 2). NLS 1 is a classical, bipartite NLS and is also present in the TRβ1 isoform. NLS2 is a conserved, monopartite NLS that is only present in the TRα1 isoform. It has been previously demonstrated that both NLS are individually capable of directing GFP-GST-GFP (G3)-tagged domain constructs to the nucleus, though NLS-2 is less efficient.

These same G3 domain constructs were used to investigate binding of importin α1, β1, and 7 to both TRα1 NLS. GFP-Trap co-immunoprecipitation (Chromo-Tek) and immunoblotting techniques, we have demonstrated that the importin α1/β1 heterodimer interacts with both the A/B and Hinge domains, while Importin 7 interacts only with the A/B domain. This is consistent with our findings that IPO 7 does not interact with TRβ1, which lacks NLS2.This, along with knockdown experiments, indicate that nuclear import of TRα1 involves multiple import pathways.

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Abbreviations

Kapβ: karyopherin β; family includes importins and exportins
DBD: DNA binding domain
LBD: Ligand binding domain
NES: nuclear export sequence
NLS: nuclear localization signal
NPC: nuclear pore complex
TR: thyroid hormone receptor

I. Introduction

Overview

Thyroid hormone receptors, or TRs, mediate action of thyroid hormone in the cell. These receptors act as transcription factors that modulate gene expression in response to the presence of thyroid hormone. In order to carry out its function, TR must be transported into the nucleus after translation in the cytoplasm and bind to DNA. Additionally, while TR is primarily localized to the nucleus in a healthy cell, it has been shown to shuttle in and out of the nucleus rather than simply remain there (Bunn et al. 2001; Mavinakere et al. 2012). Transport between the nucleus and cytoplasm is targeted by sequences of amino acids called nuclear localization sequences (NLSs) and nuclear export sequences (NESs), which mark molecules for nuclear import and export respectively. This transport is mediated by molecules collectively referred to as β-karyopherins, or Kapβs. Kapβs bind to proteins at NLSs or NESs and direct them toward the nucleus. The purpose of this study was to explore the precise mechanisms of nuclear import of TR.

Thyroid hormone

The thyroid hormones, T3 and T4, are essential in many bodily processes, including neurological development and metabolic regulation. There are two main forms of thyroid hormone, thyroxine (T4) and triiodothyronine (T3). T3 is the active hormone, and T4 must be converted to T3 by deiodinases before acting on the cell (Chiamolera and Wondisford 2011; Zhang and Lazar 2000).

The hypothalamus secretes thyrotropin releasing hormone, or TRH, which stimulates the anterior pituitary to release thyroid-stimulating hormone (TSH). TSH in turn stimulates the thyroid gland to produce thyroid hormones T3 and T4. Levels of T3 and T4 must be kept within a relatively narrow range, and this system, called the hypothalamus-pituitary-thyroid (HPT) axis, is tightly regulated by a negative feedback mechanism. Increased levels of T3 and T4 inhibit the production of TSH by the anterior pituitary as well as the production of TRH by the hypothalamus (Chiamolera and Wondisford 2011) (Fig. 1).

Abnormally low levels of thyroid hormone, a physiological condition known as hypothyroidism, can lead to a variety of diseases and conditions. During fetal development, low levels of thyroid hormone in the mother can lead to mental retardation, cerebral spastic diplegia, and other conditions even in the absence of other symptoms of hypothyroidism. Hypothyroidism in neonates can lead to less severe mental retardation as well as growth retardation and speech deficits (Williams 2008). In adults, abnormal levels of hormone can lead to disorders of metabolism as well as neurological symptoms such as depression (Hage and Azar, 2012).

It was previously believed that thyroid hormone could diffuse passively into the cell in a manner similar to steroid hormones. However, it has since become clear that transport of thyroid hormone into cells is facilitated by dedicated transport proteins. These thyroid hormone transporters, such as monocarboxylate transporter 8 (MCT8), are essential for proper function of thyroid hormone. Inactivating mutations of these transport proteins can lead to profound mental retardation and a condition called Allan-Herndon-

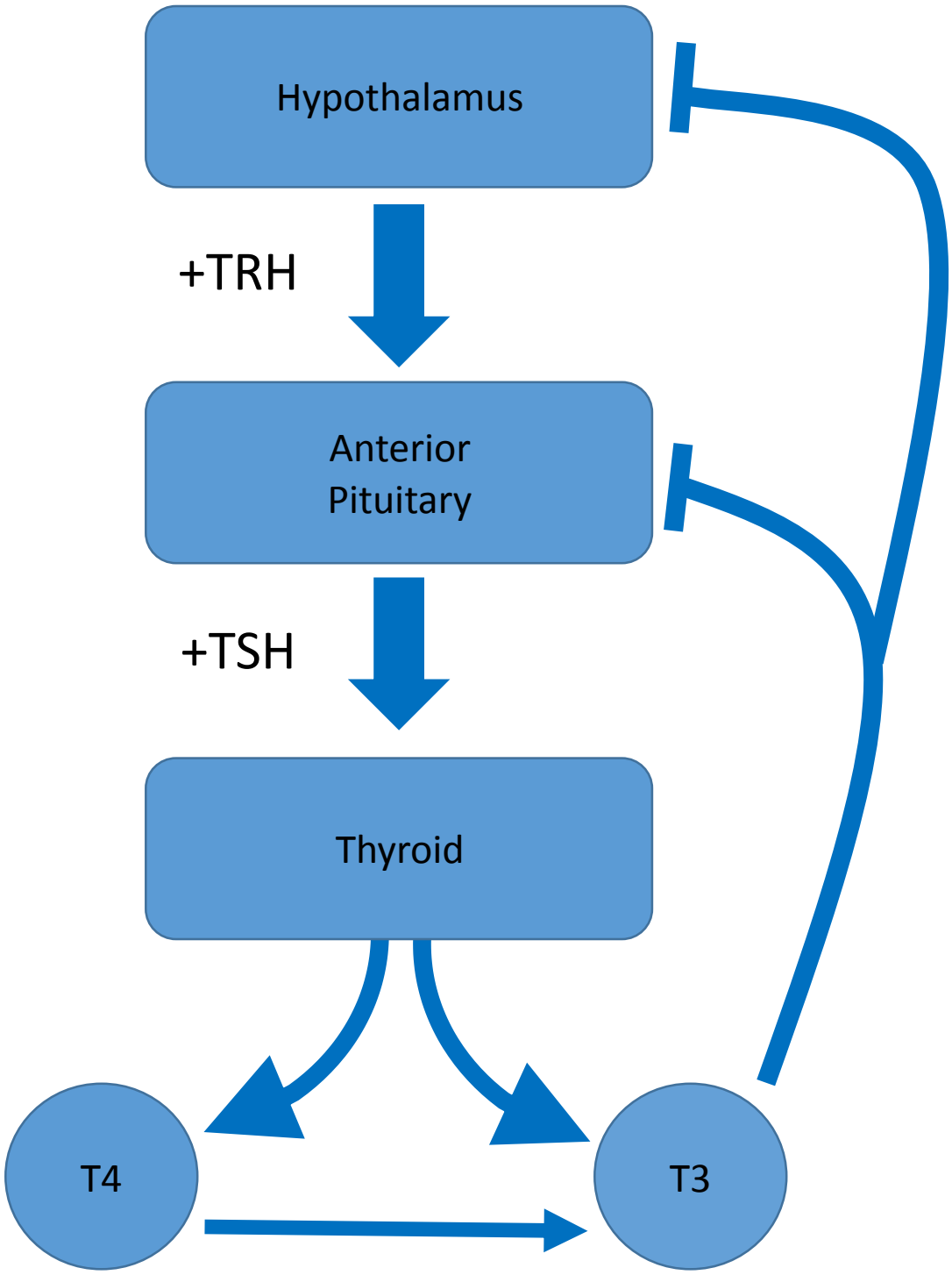

Fig. 1. The hypothalamus-pituitary-thyroid (HPT) axis. Secretion of thyrotropin releasing hormone (TRH) by the hypothalamus stimulates the anterior pituitary to release thyroid stimulating hormone (TSH), which in turn simulates the thyroid to produce the thyroid hormones T4 and T3. T4 must be converted to T3 by iodinases to be active.

Dudley syndrome (Brent 2012; Heuer and Visser 2009). Different transporters can preferentially transport T4 or T3 (Heuer and Visser 2009).

Thyroid hormone receptor: mechanism of action

TR and gene expression

Thyroid hormone affects gene expression through the thyroid hormone receptor, or TR. TR is encoded by two genes, *TRHA* and *TRHB*, which encode the α and β isoforms respectively. TR isoforms are expressed in different amounts in different tissues; TRα, the focus of this thesis, is expressed primarily in the brain, heart, and skeletal muscle (Brent 2012; Cheng et al. 2010). Differential splicing of each isoform results in a number of splicing variants: TRα1, TRα2, TRα3, TRβ1, TRβ2, and TRβ3. TRα2 and TRα3 are unable to bind T3, whereas all of the TRβ isoforms are capable of binding T3 and are expressed at different levels in different tissues (Brent 2012).

TR is a nuclear receptor, part of a superfamily of transcription factors which control gene expression in response to binding of their cognate ligand. Within this superfamily are three types of receptors: type I, which bind ligand in the cytoplasm and are transported into the nucleus after ligand binding; type II, including TR, which are retained in the nucleus and bind DNA in both the presence and absence of ligand; and type III, which includes the "orphan receptors". Type II receptors are capable of both activating gene transcription in the presence of ligand and repressing transcription in the absence of ligand, and so reside in the nucleus. "Orphan receptors" are receptors whose ligands are not known (McKenna and O'Malley 2002). TR is a type II receptor, and localizes to the nucleus and binds to DNA regardless of the presence of thyroid hormone.

TR acts by binding to DNA at TREs, or thyroid hormone response elements. When ligand is absent, TR generally silences gene transcription; when ligand is bound, it induces a conformational change that promotes gene expression. Most TR functions as a heterodimer with the RXR, though homodimers and monomers are also able to bind DNA (Zhang and Lazar 2000). Both TR's activating and repressive functions are physiologically important; elimination of TR altogether results in different phenotypes than depletion of T3 in mice. Additionally, in some species, TR is expressed in early development before production of thyroid hormone begins. These factors indicate that TR's actions in the absence of ligand – i.e., suppression of certain genes – are physiologically relevant in addition to its positive regulation functions (Bernal and Morte 2013).

Mutations in TR have been linked to diseases such as resistance to thyroid hormone (RTH) and cancers. Resistance to thyroid hormone, due to dominant-negative mutations in TRβ is best characterized. Until recently, RTH was thought to only emerge from mutations to TRβ and TRα mutations were thought to be lethal (Brent, 2012; Espiard et al. 2015; Ortiga-Carvalho et al. 2014). RTH due to TRβ mutations can result in many different phenotypes; symptoms are more severe in individuals who are homozygous for a dominant-negative mutation. These symptoms include goiter, learning disability, delays in growth and development, and hearing deficits (Brent 2012). RTH due to dominant-negative mutations in TRα can result in delays in bone development, short stature, and cognitive impairment (Brent 2012; Espiard et al. 2015; Ortiga-Carvalho et al. 2014).

TR and functional domains

TR, like all nuclear receptors, is comprised of four functional domains: the N-terminal A/B domain (also called the AF1 domain), the DNA binding domain (DBD), the Hinge domain, and the ligand binding domain (LBD) (Fig. 2). While the DBD, Hinge domain, and LBD are similar across nuclear receptors and between TR isoforms, the A/B domain remains variable. The region C-terminal to the LBD also shows variability across receptors (Mavinakere et al. 2012). Each domain has an individual function and is capable of acting outside of the context of the entire protein or when inserted into another protein (Zhang and Lazar, 2000).

The A/B domain, also called the AF1 domain, is important in activating gene transcription. This domain is also where the structure of TRα and TRβ differ the most (Brent 2012). Isoform-specific actions of TR are likely due to variations in this region, including the higher potency of TRα (Hollenberg et al. 1995). In TRα, the A/B domain contains a nuclear localization signal (NLS-2) that is absent in TRβ (Mavinakere et al. 2015). The DBD, which contains two zinc finger motifs, recognizes TREs on DNA and directly interacts with these elements; this domain is highly conserved across nuclear receptors (Cheng et al. 2010; Brent 2012; Zhang and Lazar, 2000). The Hinge domain connects the DBD and LBD. It also contains a nuclear localization signal, designated NLS-1; this localization signal is present in both TRα and TRβ (Mavinakere et al. 2012).

The LBD is the largest single domain in TR. In the absence of the receptor's ligand, T3, the LBD is involved in the recruitment of corepressors which silence gene expression. Upon T3 binding to the LBD, the receptor undergoes a conformational change which results in recruitment of coactivators, leading to an increase in gene

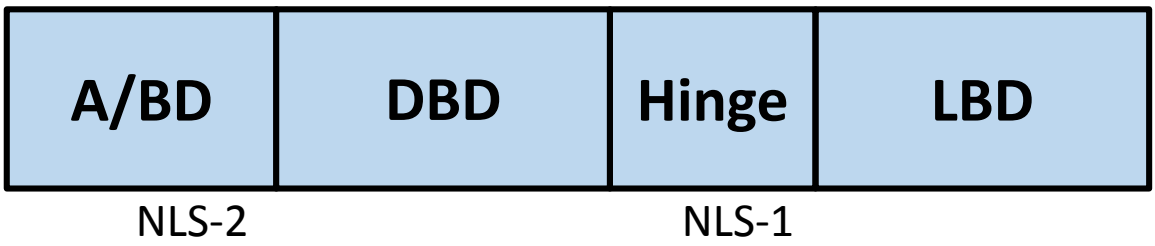

Fig. 2: Schematic diagram of domains of TRα1. Localization signals are labeled; export sequences are omitted. Specific locations of NLS: NLS-1 stretches from residues 130-147, in the Hinge region. NLS-2 stretches from residues 22-29, in the A/B domain.

transcription. This means that TR has a dual role as both an activator and repressor of transcription, and both roles have been shown to be important in development (Brent 2012; Zhang and Lazar 2000; Bernal and Morte 2013). It has been shown in some cases that TR can activate gene expression in the absence of T3 and repress transcription upon ligand binding, but generally ligand binding is necessary for gene activation. The LBD is also involved in formation of heterodimers with other nuclear receptors including RXR. The LBD is truncated in TRα2, an alternative splicing product of the TRα gene. Unable to bind ligand, TRα2 acts as a dominant-negative repressor of gene transcription (Zhang and Lazar 2000; Brent 2012).

Nucleocytoplasmic transport, intracellular localization, and TR function

Overview of nuclear import and export

Proper cell function is dependent on efficient, selective shuttling of molecules between cellular compartments. The ability of molecules to travel into and out of the nucleus is particularly essential: RNA transcripts of genes must be transported out of the nucleus for translation, and translated proteins that function in the nucleus must be

transported into it. Nuclear pore complexes (NPCs) are large, multiprotein structures embedded in the nuclear membrane and act as selective gates through which proteins can be translocated. The NPC is made up of approximately 30 proteins called nucleoporins. Small proteins (less than ~40 kDa) can diffuse passively through the complex; however, larger proteins must be transported actively into the nucleus (Marfori et al. 2010).

Transport into and out of the nucleus is facilitated by a family of proteins called karyopherin β proteins, or Kapβs (Cook et al. 2007; Chook and Suel 2010). There are over 20 human Kapβ proteins known (Cook et al. 2007; Chook and Suel 2010). This family can be divided into importins and exportins, which, as their names imply, regulate nuclear import and export respectively. These molecules regulate transport of protein cargo by recognizing nuclear localization signals (NLSs, for import) and nuclear export signals (NESs, for export) on target molecules. After binding their cargo, the importin:cargo complex then docks at the NPC by interacting with special nucleoporins lining the inside of the nuclear pore, which contain series of highly disordered phenylalanine-guanine (FG) repeats (Cook et al. 2007; Marfori et al. 2010; Chook and Suel, 2010). Cargo and importin/exportin are then translocated across the nuclear membrane in a process that takes milliseconds (Grunwald and Singer 2012) (See Fig. 3).

Association and disassociation of cargo and importin/exportin is controlled by Ran. (See Fig. 3). Ran is a small G protein that varies between a GTP and GDP bound state; Ran-GDP is incapable of binding Kapβ proteins. For import, association between importin and cargo occurs in the cytoplasm, with no Ran bound. Upon entry into the nucleus, Ran-GTP binds to importin and cargo dissociates. For export, cargo binds to exportin along with Ran-GTP. After transport, Ran-GTP is hydrolyzed in the cytoplasm

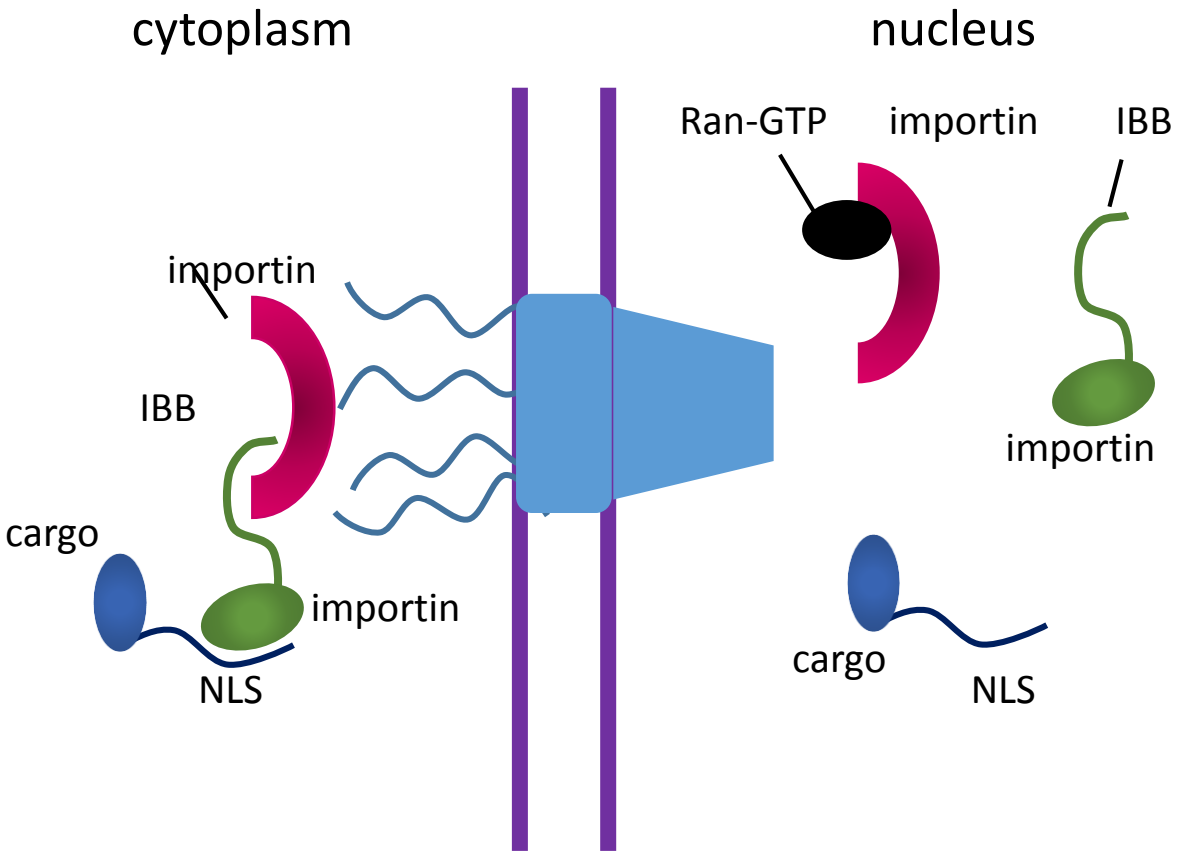

Fig 3. Schematic of the classical nuclear transport pathway. The NLS on cargo protein binds to the binding grooves of importin α. The importin β binding domain then binds to the concave surface of importin β (here represented as an arc, though the binding actually occurs at the concave surface of the superhelix). Importin β then interacts with the NPC, facilitating transport across the nuclear envelope. Ran-GTP binding induces import complex dissociation. Abbreviations: IBB: importin β binding domain.

and cargo, Ran-GDP, and the exportin dissociate (Cook et al. 2007; Chook and Suel, 2010).

Structure and function of the NPC

As described above, the nuclear pore complex is a large, supramolecular structure of about 30 different proteins. The NPC is assembled around a central pore with a diameter of 30-40 nm (Lemke 2016). It includes two coaxial rings, one each on the nuclear and cytoplasmic faces of the membrane, which form a channel. Cytoplasmic filaments are anchored to the central ring on the cytoplasmic side, while the nuclear side includes a distal ring that provides anchoring sites for a nuclear basket extending into the nucleus (Stoffler et al. 2003; Hoelz et al. 2011). The channel is filled with proteins called FG-nucleoporins, so named because they are rich in phenylalanine-glycine residues. The FG repeats of the nuclear pore are highly disordered and form a permeability barrier within the central channel (Grunwald and Singer, 2012; Lemke 2016). This makes them difficult to study, as their structure and interactions can change under a variety of conditions (Lemke 2016).

While the precise mechanism of translocation across the membrane has not been confirmed, there are several models to explain the selectivity and speed of transport. In general, models suggest that FG repeats form a physical barrier that prevents nonspecific entry or that interaction between the FG repeats and import/export complexes causes structural or chemical changes of the FG repeats. These include the “entropic exclusion” model, in which selectivity is achieved based on the volume occupied by FG repeats; the “entropic brush” model, in which interaction with import/export factors causes collapse of the FG repeats and allows cargo to go through the channel; models in which

interaction with transport factors causes polymerization of FG repeats, forming a "selective gel phase"; and a slide-and-exchange model in which FG repeatedly transitions between strongly and weakly interacting with import/export factors, and displacement of FG repeats by competing repeats while in the weakly interacting state (Grunwald and Singer 2012; Raveh et al. 2016). This causes cargo to "slide" through the NPC channel. It may be true that multiple models are used or contribute to nucleocytoplasmic transport, though certain modes would be more common than others (Raveh et al. 2016).

Control of nuclear transport: rate and specificity

Rates of nuclear import or export are regulated by a variety of mechanisms. Several of these involve increasing or lowering the affinity of NLSs or NESs to bind to importins or exportins. This can be achieved by post-translationally modifying these sequences or the protein itself, such as by phosphorylation (Christie et al. 2015). Other modes of regulation involve masking the NLS or NES so Kapβ proteins cannot bind (Christie et al. 2015). Conformational changes in cargo proteins can reveal or hide NLSs or NESs. Rate of import can also be regulated by modifying importins, rather than cargo; for example, acetylation of importin α in the importin β binding domain (see "The Classical Nuclear Import Pathway") increases its affinity for importin β1 *in vitro* (Christie et al. 2015).

Different Kapβ factors can recognize and transport different cargo. Many different NLSs and NESs have been discovered and characterized, and many different nuclear import and export pathways have been characterized as well (Mavinakere et al. 2012; Cook et al. 2007; Chook and Suel, 2010). Dysfunction of import or export pathways can have physiological effects. Exportins are necessary for the nuclear export

of mRNA and thus protein expression (Cook et al. 2007). In the yeast *Saccharomyces cerevisiae*, the importin β homolog Kap95p has been shown to be important in cell cycle progression (Christie et al. 2015). In mouse, importin-13 is important in the progression of meiosis (Kimura and Imamoto 2014). Nuclear import and export are essential to the function of many biomolecules.

As described above, TR functions in the nucleus by binding to DNA; however, it must be imported into the nucleus after being translated in the cytosol. Additionally, though TR functions by binding to DNA in the nucleus, previous research has shown that the receptor actually shuttles rapidly between the nucleus and cytoplasm (Bunn et al. 2001; Grespin et al. 2008). That is, though TR overall localizes to the nucleus and has NLSs directing that localization, it also includes NESs and is exported out of the nucleus as well. As described above, TRα1 has been shown to have two NLSs, one each in the A/B domain and Hinge domain (Fig. 2). TRα1 also has at least 3 NESs located in the LBD (Mavinakere et al. 2012; Subramanian et al. 2015).

TR localization depends on a balance between nuclear import and export. The exact function of receptor shuttling is not clear, but may have to do with receptor turnover or with mediating crosstalk with other nuclear receptors (such as RXR) (Bonamy and Allison, 2006). Disruption of this balance could be linked to disease states such as cancer (Bonamy et al. 2005; DeLong et al. 2004; Bonamy and Allison 2006). The following is a review of common nuclear import pathways, with a focus on importins α, β1, and 7, as those have been shown to be involved in nuclear import of TRα1 (Roggero et al. 2016)

Nuclear localization signals (NLSs)

The first nuclear localization signals (NLSs) were characterized in the 1980s, based on studies of the SV40 large T-antigen and *Xenopus* nucleoplasmin (Marfori et al. 2010). These are considered classical NLSs, and utilize the classical nuclear import pathway (described in detail below). Classical nuclear localization signals can be monopartite or bipartite. Monopartite signals consist of a short cluster (3-5 residues) of basic amino acids. Bipartite signals also include that cluster of basic amino acids, but differ from monopartite NLSs in that they include an additional cluster of lysine and arginine residues about 10-12 amino acid residues away (Cook et al. 2007). There is some evidence that longer linker regions are also possible (Lange et al. 2010).

Classical NLSs are recognized by importin α. They compete with the SV40 NLS for binding to importin α, because they utilize the same binding site(s) on importin α (see below, "The Classical Nuclear Import Pathway"). Other, non-classical NLSs have also been characterized. These can differ in the length of the sequence or in which importins recognize the sequence. NLS domains have also been observed that are much larger than classical NLSs and lack basic amino acids, such as the M9 NLS. In the cases of very large NLS domains, it is possible that the three-dimensional structure of the domain is crucial for proper function (Pemberton and Paschal 2005). Additionally, importin α has also been shown to bind to non-classical NLSs, though at different locations on importin α (see below).

The classical nuclear import pathway

In this pathway, importin α acts as an adaptor protein for importin β1 by binding to the NLS on the cargo protein. Importin β1 then binds to the importin β1 binding domain (IBB1) on importin α. Importin β1 then interacts with the NPC, and facilitates transport across the nuclear membrane. In some species, there are multiple variants of importin α, which can be specific for certain cargoes, but share similar structures and mechanisms of action (Christie et al. 2015). The focus of this thesis is on importin α1, as that has been implicated in TRα1 import (Roggero et al. 2016).

Recognition and binding of classical NLS by importin α occurs at the inner surface of importin α, in the NLS binding domain. This domain is comprised of up to 10 armadillo (ARM) repeats, each of which contains about 40 amino acids and forms three α-helices. These repeats then stack onto one another, resulting in a long, twisted molecule with inner concave and outer convex surfaces. NLSs bind to the inner concave surface, which includes two binding pockets of conserved tryptophan residues. These interact with the positively-charged residues of the NLS (Cook et al. 2007; Xu et al. 2010; Christie et al. 2015). The binding pockets form two binding sites, a major and minor site. Monopartite classical NLSs bind to the major site, while bipartite classical NLS bind to both the major or minor binding sites (each site interacting with one basic cluster in the bipartite signal). The linker region accounts for the distance between the two binding sites (Cook et al. 2007; Nakada et al. 2015) (Fig. 4).

For transport to occur, importin β1 must bind to importin α. Importin β1 includes about 19 HEAT repeats. These repeats consist of approximately 40 amino acids each and

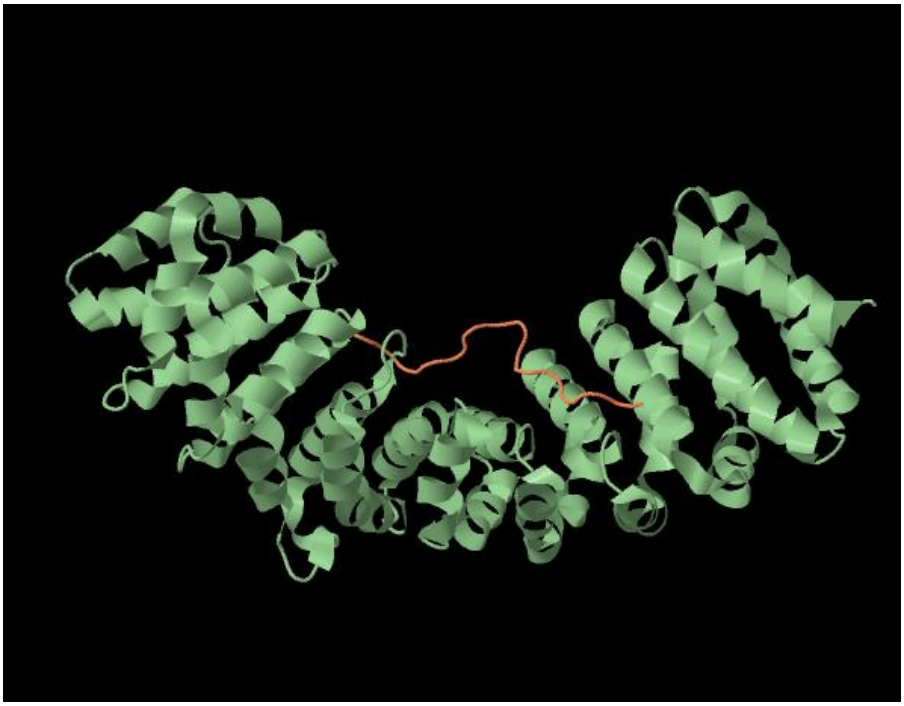

Fig 4. Structure of mouse importin α bound to nucleoplasmin, a classical bipartite NLS. Nucleoplasmin NLS is shown in orange. (Marfori et al. 2012). PDB ID: 3UL1

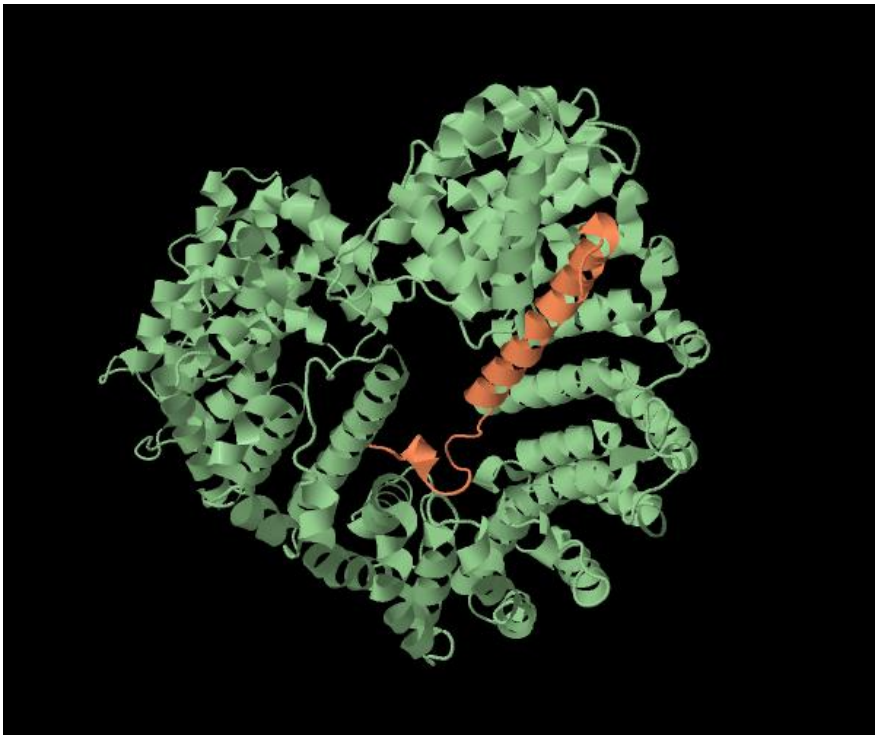

Fig 5. Structure of importin β bound to the IBB of importin α. (Cingolani et al. 1999). PBD ID: 1QGK

are so named because they were first discovered in the **H**untingtin, **e**longation factor 3, PR65/A subunit of protein phosphatase 2**A** and the **T**OR lipid kinase. The repeats stack to form a superhelical structure; similarly to the importin α NLS-binding domain, this creates concave and convex surfaces. Cargo molecules, in this case importin α, bind to the concave surface of the superhelix (Cook et al. 2007; Cingolani et al. 2002; Marfori et al 2010). Importin β1 binds to the IBB domain of importin α by wrapping closely around it (Cook et al 2007; Marfari et al 2010; Xu et al. 2010; see Fig. 5). The convex surface of the superhelix then interacts with the FG-repeats of the NPC.

Non-classical nuclear import pathways

Recognition of non-classical NLSs by importin α

Importin α can also recognize non-classical NLSs. Some can bind directly to the minor binding site and do not use the major binding site, though these are rarer than classical NLSs (Nakada et al 2015, Chang et al 2013; Kosugi et al. 2008). Kosugi et al. (2008) described six classes of NLSs that interactwith importin α, two of which bound exclusively to the minor site. These classes of NLSs consist of a basic cluster followed by C-terminal hydrophobic amino acids (Christie et al. 2015).

Recognition of cargo proteins by importin β1 alone

Importin β1 can also bind to cargo directly, without the need for an adaptor. Proteins that are transported by importin β1 alone differ significantly from each other, and NLSs can vary in size and charge of residues (Marfori et al. 2010; Cingolani et al. 2002). This is partially due to the large surface area of the protein and its flexible structure (Marfori et al. 2010). Several proteins, including parathyroid hormone related

protein (PTHrP) and the sterol-regulatory element binding protein 2 (SREBP-2), have been shown to be importin α independent and interact with importin beta1 (Cingolani et al. 2002). PTHrP binds at a separate but overlapping cargo binding site from that which binds to the IBB domain of importin α (Cingolani et al 2002). Importin β1 must adopt a more open conformation to bind to SREBP-2, and binding relies on hydrophobic interactions rather than the electrostatic interactions necessary for IBB domain binding to importin β1 (Marfori et al. 2010).

Importin 7

Importin 7 can facilitate transport as a monomer or as heterodimer with importin β1. (Chook and Suel 2010, Roggero et al. 2016). Importin 7 is also involved in the transport of the glucocorticoid receptor (GR), another member of the nuclear receptor superfamily (Friedman and Yamamoto 2004). Generally, importin 7 appears to recognize a diverse array of localization sequences (Chook and Suel 2010). Molecules imported by importin 7 often can utilize other importins to enter the nucleus, including the α/β heterodimer pathway or importin β alone (Chook and Suel 2010).

Nuclear import of thyroid hormone receptor α: thesis objective

TRα1 has been demonstrated to have two NLSs: one classical, bipartite NLS located in the Hinge region, called NLS-1, and a novel, monopartite NLS in the A/B domain, called NLS-2 (Mavinakere et al. 2012; Fig. 2). The minimal amino acid sequence for NLS-1 is ^{130}KRVAKRKLIEQNRERRRK^{147}; the minimal amino acid sequence for NLS-1 is ^{22}PDGKRKRK^{29} (Mavinakere et al. 2012). NLS-2 is absent in the TRβ1 isoform and is not active in TR's oncogenic form, v-ErbA (Mavinakere et al.

2012). This may contribute the slightly greater cytoplasmic localization of TRβ and the cytoplasmic localization of v-ErbA (Mavinakere et al. 2012).

NLS-2 has been shown to be necessary for efficient nuclear localization of TRα1. A mutation that disrupts NLS-2 results in less nuclear retention of TRα1, causing its localization to become more cytosolic (Mavinakere et al. 2012). Though NLS-1 in TRβ is sufficient to facilitate nuclear import, it is unable to fully compensate for the loss of NLS-2 in TRα1 (Mavinakere et al. 2012). Additionally, amino acids flanking the minimal NLS-2 sequence can have profound effects on its efficiency (Mavinakere et al. 2012). In chicken TRα1, the first 11 N-terminal amino acids are necessary for complete nuclear localization; though these amino acids do not overlap directly with NLS-2, this demonstrates the importance of the A/B domain in directing nuclear localization of TRα1 (Andersson and Vennström, 1997).

Mislocalization of TR may be linked to oncogenic conversion of cells. v-ErbA, an oncogenic form of TR, has an inactive NLS-2 and an acquired viral nuclear export sequence (DeLong et al. 2005). v-ErbA maintains a significant cytosolic population in the cell (Bonamy and Allison 2006). v-ErbA dimerizes with TRα1 and RXR; this dimerization, combined with the cytosolic localization of v-ErbA, prevents a subpopulation of the receptors from entering the nucleus and results in a significant portion of endogenous receptor to remain the cytoplasm (Bonamy and Allison 2005).

Knockdown of importins β1, 7, and α1 all result in decreased nuclear localization of TRα1. Inhibition of importin β1-mediated transport with importazole, a specific inhibitor of importin β1, also lead to a reduction in nuclear localization. This indicates that TRα1 uses these importins for nuclear import. Inhibition of importin β1 with

importazole also resulted in decreased nuclear localization of TRβ1; because TRβ1 and TRα1 share only the classical NLS in the Hinge region, this suggested that NLS-1 in the Hinge domain is involved in importin-β1 mediated transport (Roggero et al. 2016). Knockdown of other importins, including importins 4, 5, and 8, had no effect on nuclear localization of TRα1. Knockdown of variants of importin α (α2, α3, etc.) also had no effect on localization (Roggero et al. 2016).

Importins β1, 7, and α1 have been shown to coimmunoprecipitate with GFP-tagged TRα1 transfected into HeLa cells. This demonstrates that these importins interact with TRα1 either directly or as part of a complex (for example, the importin α/β1 heterodimer) (Roggero et al. 2016). Importin 7 does not coimmunoprecipitate beyond background levels with TRβ1, suggesting that importin 7 does not interact with the Hinge domain NLS-1.

The main objective of this thesis research was to investigate importin binding to TRα1 NLSs, and specifically to determine if different importins interact specifically with either NLS. Fully understanding TR's nuclear localization pathways is important in understanding TR's overall mechanism of action. This thesis focuses on the nuclear import of TRα1 and its interaction with import proteins.

II. Methods

See Appendix 1 for the composition of all mentioned reagents.

Subcloning

Initially, in order to investigate binding between TRα1's nuclear localization signals and various importins, we attempted to generate His_6 and GST (glutathione-S-transferase; see glossary)-tagged constructs of each domain of TRα1, using the Gateway cloning system (Thermo Scientific).

The Gateway cloning system uses site-specific recombination by topoisomerase I and involves cloning an insert into an entry vector before eventually cloning it into final expression vector (also called a "destination vector"). Individual domains and combinations of domains of TRα1 were amplified from rat TRα1 by PCR using specially designed primers. These primers contained sequences that would insure that the PCR product would be cloned into the entry vector in the correct orientation. The constructs were as follows: A/B domain, A/B-DBD, DBD alone, DBD-Hinge, Hinge alone, and LBD. After amplification by PCR, the inserts were cloned into the pENTR/D-TOPO vector by site-specific recombination. The insert was then cloned into a destination vector using the system's LR Clonase Mix, which contains a mixture of integrase, integration host factor, and excisionase enzymes.

These attempts were not successful, and resulted in no verifiable protein expression constructs. We found that the domains were never inserted into the entry vector or destination vector, or they could not be verified to be in the correct orientation in the destination vector.

We then attempted to generate clones using more traditional subcloning methods. We made use of GeneArt plasmids containing synthetic genes encoding the A/B domain, Hinge domain, and the full TRα1 (Life Technologies). Two expression vectors were used, the pGEX-6P2 (GE Healthcare) and the pQE-30 Xa (Qiagen) for the GST and His6 tags respectively. Plasmids were sequentially digested using BamHI and SmaI restriction enzymes at appropriate temperatures; a variety of reaction times were attempted, ranging from 1 hour to over 5 hours. Between restriction digests, reactions were purified using the Qiagen PCR Purification Kit. After the second digest, samples were run on a 1% or 2% agarose gel according to size of the fragment of interest. Fragments were then excised from the gel and purified using Qiagen Gel Purification Kit.

Several attempts at ligation reactions were made, experimenting with the relative ratios of insert to vector. Regardless of ratio, all reactions were ligated using New England Biolabs T4 ligase and incubated at 16 degrees Celsius for 12-16 hours. This resulted in some clones that could be verified by sequencing (His_6-Hinge, His_6-TRα). However, the clones have not yet been expressed in *E. coli* and several constructs have not yet been cloned. The other constructs have not yet been verified by sequencing.

Immunoblotting

Though these cloning efforts have been somewhat successful at generating tagged protein constructs, we decided to try another approach to investigate importin binding to TRα1. We used G3 (GFP-GST-GFP)-tagged protein constructs which had already been generated for a previous study (Mavinakere et al. 2012).

HeLa cells were seeded at 9 x 10^5 cells per plate in 100 mm culture plates with MEM (10% FBS). After 24 h, cells were transfected with 10 μg plasmid using Lipofectamine 2000 and incubated at 37˚C for 26 h. Cells were then washed with Dulbecco's phosphate-buffered saline (D-PBS). Cells were treated with 0.7 mL 0.25% trypsin and collected with 1.0 mL MEM into 2.0 mL microcentrifuge tubes. Cells were then washed again with D-PBS, lysed with lysis buffer and protease inhibitor, and incubated on ice for 30 mins with mixing every 10 min.

Samples were then centrifuged at 16,000 × *g* for 10 min and the supernatant transferred to a fresh 1.5 mL tube. After dilution with 0.3 mL of Dilution/Wash Buffer, GFP-Trap agarose beads (Chromo-Tek) were added to the sample after being equilibrated using Dilution/Wash Buffer. Samples were incubated for at least 2.5 h at 4˚C with constant inversion. Afterward, samples were centrifuged at 4˚C, 3,000 *g*, for 4 min. Samples of supernatant (unbound proteins) were taken and diluted in equal volume of 2x Sample Buffer. Beads were then washed 3-4 times with Dilution/Wash Buffer, and then were resuspended in 100 μL of 2x Sample Buffer.

Samples of unbound and bound proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting. Accurate protein size was confirmed by comparison with pre-stained Kaleidoscope Protein Size Standards (Bio-Rad). Antibodies used: anti-GFP (Santa Cruz), 1:2000; anti-IPOα1 (Abcam), 1:1000; anti-IPOβ1 (Santa Cruz) 1:1000; anti-IPO7 (Abcam), 1:1000; horseradish peroxidase conjugated (HRP-conjugated) donkey anti-rabbit (GE Healthcare Life Sciences), 1:25,000.

III. Results

Importin α1 and β1 interact with both NLS-1 and NLS-2

To determine interaction between importins and different NLSs, we made use of G3 (GST-GFP-GST)-tagged protein constructs of TRα1, A/B domain, and Hinge domain. Both G3-Hinge and G3-A/B have been shown to localize to the nucleus, though G3-A/B to a lesser degree. This may indicate that NLS-2 is less efficient at directing nuclear import out of context of the whole protein (Roggero et al. 2016; Mavinakere et al. 2012).

HeLa cells were transfected with the G3-constructs as well as empty G3 vector. Cells were then lysed and immunoprecipitated using GFP-Trap. Samples were analyzed via Western blot using GFP-specific antibodies to ensure successful transfection. Next, we sought to determine if endogenous importins were binding to the exogenous constructs. On separate blots, samples were analyzed using anti-importin α1 and β1 antibodies (Fig 6 & 7). Results show that importin α1 and β1 were coimmunoprecipitated with G3-Hinge and G3-A/B, as well as full-length TRα1. This demonstrates that each NLS interacts with both of these importins.

Importin 7 may interact with NLS-2, but direct interaction was not observed

Western blots were also conducted using anti-importin 7 antibodies. No consistent, direct interaction between either NLS and importin 7 was observed (Fig. 8). This was puzzling, because importin 7 was shown to be involved in TRα1 transport, and to not bind to NLS-1 based on experiments with TRβ1 (Roggero et al. 2016). It may be possible that NLS-2 is not displayed properly or may be unable to bind to importin 7 out

of the context of the full-length protein. Further investigation will be necessary to determine the role of importin 7 in TRα nuclear import.

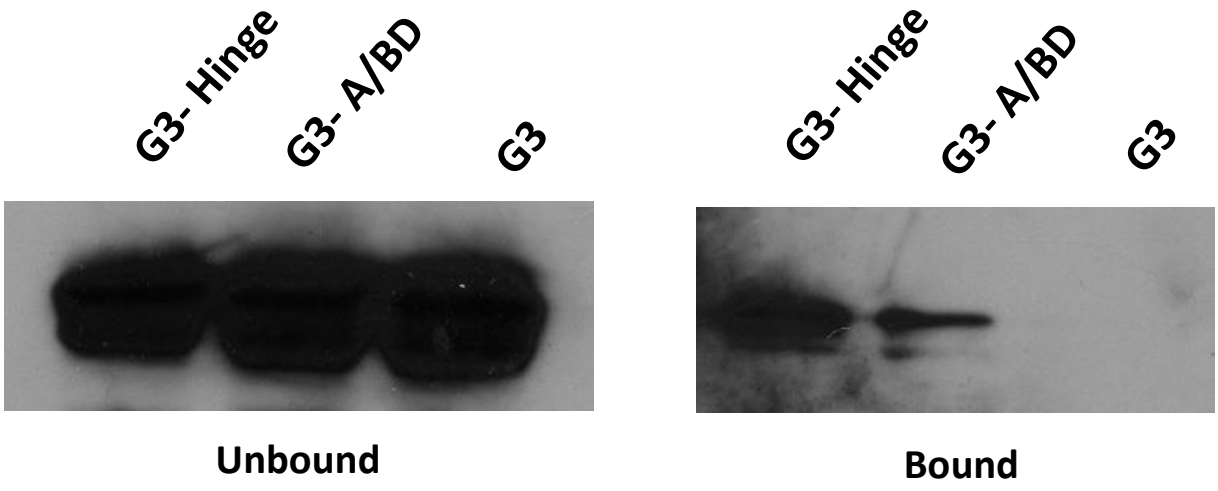

Fig. 6. Binding of importin α1 to TRα1 domains. Blots were analyzed using anti-importin α1 antibodies (Abcam). Binding was observed between importin α1 and the Hinge and A/B domains. Empty G3 vector included as negative control.
Exposure times: Unbound: 5 s Bound: 4 min

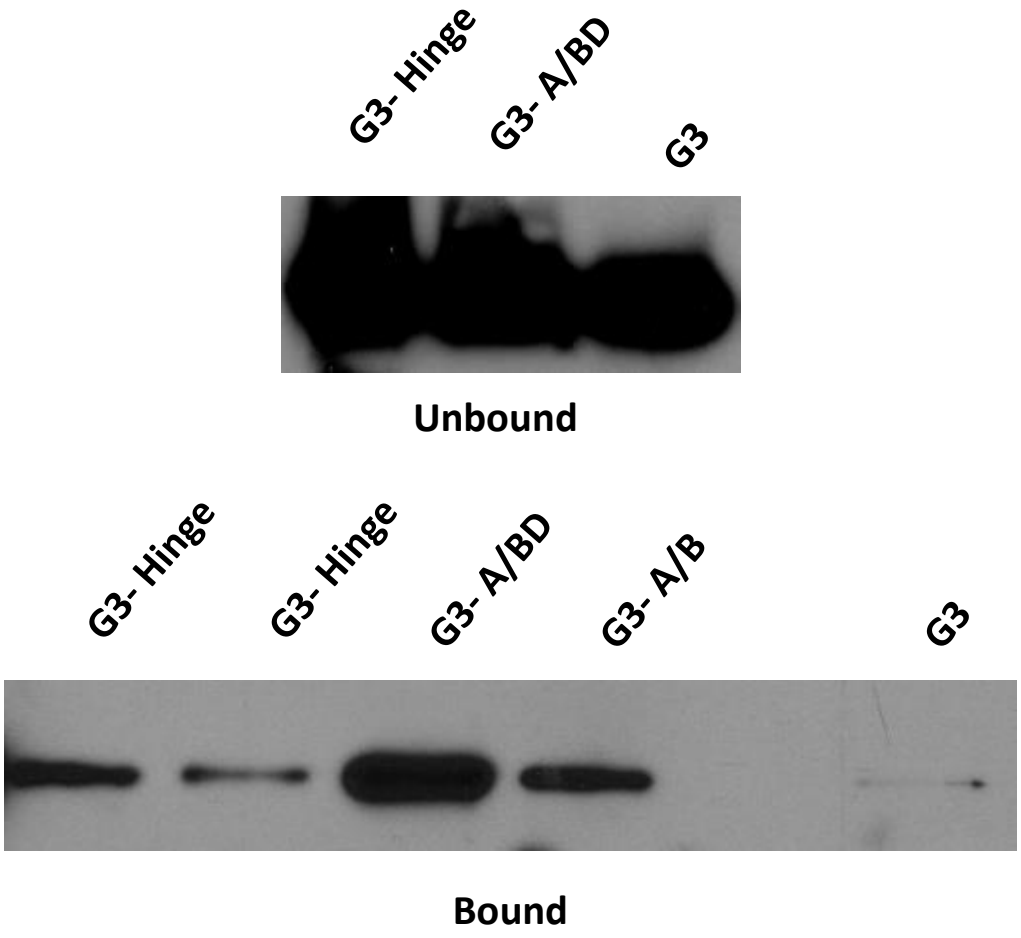

Fig. 7. Binding of importin β1 to TR α1 domains. Blots were analyzed using anti-importin β1 antibodies (Santa Cruz). Each lane indicates a separate experimental replicate. Binding was observed between importin β1 and the Hinge and A/B domains. Empty G3 vector included as negative control.
Exposure times: Unbound: 15s; Bound: 1 min

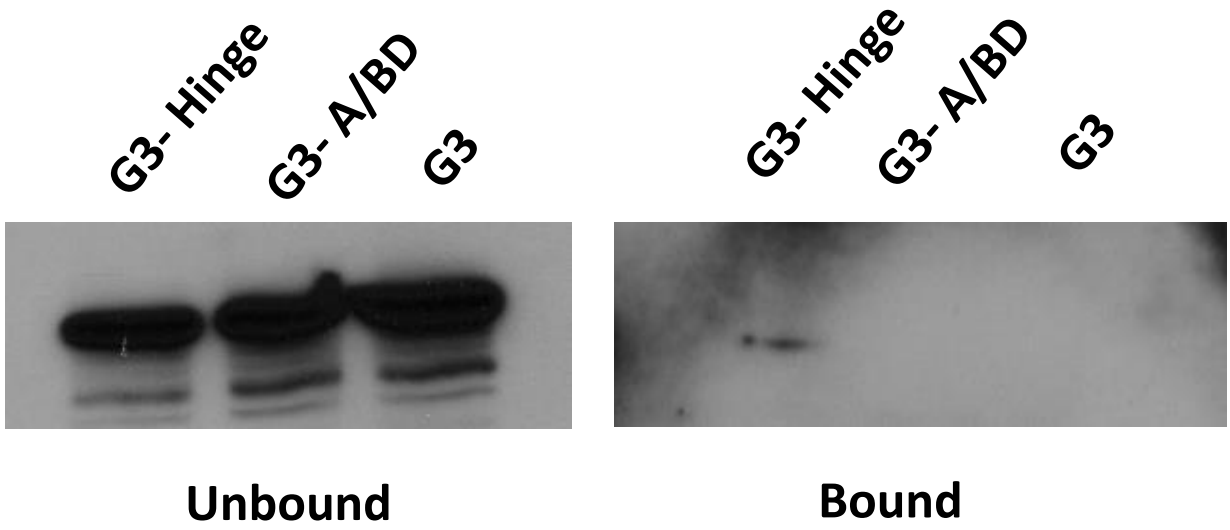

Fig. 8. No consistent interaction between importin 7 and TRα1 domains was observed. Interaction between either the A/B or Hinge domains and TRα1 was not shown above background levels. Blots were analyzed using anti-importin 7 antibodies (Abcam). G3 included as negative control.
Exposure times: Unbound: 5s; Bound: 4 min

IV. Discussion & Future Directions

This study, combined with past work, has shown that TRα1 utilizes multiple pathways to enter the nucleus. Direct interaction between both TRα1 NLS and importins α and β has been demonstrated, but further investigation is necessary to determine interaction between importin 7 and TRα1. More work is also necessary to determine if importin 7 is capable of transporting TRα alone or if it does so as part of a heterodimer with importin β. This work isf important in understanding the nature of TR shuttling between the nucleus and cytoplasm, which will help to understand the biological function and effects of receptor shuttling.

Function of receptor shuttling

The exact purpose of receptor shuttling has not yet been fully explained. It is possible that shuttling out the nucleus is necessary for degradation and turnover of TR. It is also possible that shuttling contributes an additional level of transcriptional regulation by controlling the amount of TR in the nucleus at a given time. For example, v-ErbA, an oncogenic form of TRα, localizes to the cytosol and forms dimers with wild-type TRα. This dimerization may maintain a subpopulation of wild-type TRα in the cytoplasm, which would negatively impact TRα's ability to regulate gene expression (Bonamy and Allison 2005). However, non-genomic actions of TRα or forms of TR have also been observed outside of the nucleus, and may begin to explain the function of TR shuttling and the role multiple TRα NLS play in TRα function.

A truncated form of TRα (TRΔα) is involved in mediating actin polymerization in the cytoplasm in response to T4 binding (Davis et al. 2016; Cheng et al. 2010). This form is comprised of only the C-terminal portion of the LBD and lacks any NLS (Davis et al. 2014). p30-TRα, another truncated form of TRα transcribed from an internal state site, is located at the nuclear membrane, and is important in inducing cellular proliferation by interaction with signal transducing proteins upon T3 binding (Davis et al. 2015; Kalyanaraman et al. 2014). Truncated TRα is also involved with mediating T3 effects on MAPK and phosphatidylinositol-3-kinase (PI3K) mediated signaling in cells (Davis et al. 2014; Kalyanaraman et al. 2014). A form of TRα lacking the A/B domain is found in the mitochondria and regulates T3-dependent gene expression there (Cheng et al. 2010). These functions, combined with the fact that TRα1 requires NLS-2 in the A/B domain for fully efficient nuclear transport (see introduction), indicates that NLS-2 could have an important role in controlling and targeting the action of TRα.

Only truncated forms of TRα have been shown to have cytoplasmic or non-nuclear function. Full-length TRα, though it shuttles between the nucleus and the cytoplasm, seems to function only in the nucleus (Cheng et al. 2010). Full-length TRβ, however, has been demonstrated to function in the cytoplasm (Cheng et al 2010; Martin et al. 2014). One such function is regulation of PI3K-mediated signaling. TRβ forms a cytoplasmic complex with PI3K, which dissociates upon addition of ligand, after which TRβ goes into the nucleus (Martin et al. 2014).

It is possible that cytoplasmic activity of full-length TRβ1 is related to the difference in localization between full-length TRβ and TRα, and for the additional NLS in TRα. Full-length TRβ maintains a larger cytoplasmic subpopulation in than full-length

TRα, indicating that NLS-2 may play an important role in efficient nuclear targeting of the receptor. This may be because full-length TRβ has important cytoplasmic functions, while only truncated forms of TRα (which may have altered or missing NLSs) have been demonstrated to act in the cytoplasm (Davis et al. 2014; Cheng et al. 2010; Martin et al. 2014; Kalyanaraman et al. 2014; Lin et al. 2009).

Significance of multiple import pathways and multiple NLSs

Many proteins, including other nuclear receptors, utilize multiple transport pathways (Friedman and Yamamoto, 2004; Christie et al. 2015). TRα's use of multiple import pathways could serve a variety of functions. The glucocorticoid receptor (GR), for example, has been shown to interact with both importin 7 and the importin α/β heterodimer for nuclear import (Friedman and Yamamoto, 2004). The androgen receptor also uses multiple import pathways (Li et al. 2013).

Proteins may utilize multiple import pathways to achieve finer control of nuclear import. For example, different pathways may be used at different times during the cell cycle, or under different conditions. Amounts of various import factors can vary between cell types (Kimura and Imamoto 2014). It is also possible for different NLSs to act in a cooperative manner, which is suggested by the fact that loss of one NLSs negatively affects TRα1's import efficiency (Mavinakere et al. 2012). Proteins may also make use of multiple pathways in order to compensate should a pathway become inhibited or if a protein must compete with another protein for use of particular pathway. In all, the fact that TRα1 uses more than one import pathway is not unusual (Roggero et al. 2016).

Many of importin 7's cargo proteins are also imported by another import pathway. The glucocorticoid receptor, as well as viral proteins such as HIV-1 Integrase, are imported by both importin 7 and the α/β1 heterodimer; further, HIV-1 integrase is imported both by importin 7 alone and by the importin 7/β1 heterodimer (Chook and Suel 2011). TRα would be one of many proteins which use importin 7 in addition to other pathways for nuclear import.

Future directions

Direct interaction between NLS-2 and TRα1 was not observed consistently. However, RNA knockdown experiments as well as *in vitro* import assays indicated that importin 7 was involved in TRα1 nuclear import. The fact that importin 7 was confirmed not to interact with NLS-1 in TRβ1 suggests that importin 7 would interact with NLS-2 (Roggero et al. 2016). Further investigation is necessary to clarify this problem, and to determine how importin 7 may be involved with TRα1 nuclear import. Additional experiments to confirm direct interaction between importin 7 and NLS-2 of TRα1 are needed, possibly using different protein constructs to insure that the NLS is properly displayed to allow importin 7 binding. Amino acids that surround NLS-2 can greatly affect the efficiency of the NLS (Mavinakere et al. 2012). As such, these experiments could also make use of full-length TR with mutations in NLS-1 that prevent importin binding, in order to keep NLS-2 in the context of the entire protein. Additionally, it is necessary to explore whether importin 7 alone is sufficient for transport of TRα or if it acts as a heterodimer with importin β1. *In vitro* pull-down assays, rather than *in vivo* experiments, will also provide further insight into interactions between importins and TR.

V. Significance and Final Conclusions

Understanding nuclear import and export of TRα is essential to understanding its overall function in a cell. TRα acts primarily as a transcription factor, and as such must enter the nucleus after translation in the cytoplasm. Nuclear retention of TRα is necessary for appropriate activation and repression of target genes (Cheng et al. 2010). Control of nuclear-cytoplasmic transport is therefore an important factor in the activity of TRα, as well as the activity of other nuclear receptors and proteins.

Mislocalization of nuclear receptors has been linked to diseases including cancer. For example, a mutation common in prostate cancer leads to increased nuclear localization of the androgen receptor independent of ligand (Li et al. 2013). In v-ErbA, an oncogenic form of TRα, acquisition of an additional NES on TRα causes it to remain cytosolic, and dimerization with wild-type TRα blocks its import into the nucleus (Bonamy and Allison 2005). Because of their role in control of gene expression, nuclear receptors in general are potential targets for drug therapies for cancers (Li et al. 2013; Bonamy and Allison 2005; Cheng et al. 2010). Fully understanding the mechanisms controlling TR localization will elucidate TR's control over gene expression and potentially reveal targets for treatment or prevention of diseases.

VI. References

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VII. Appendix I – Solutions and Reagents

Dulbecco's Phosphate-Buffered Saline (D-PBS)

0.1 g	KCl
0.1 g	KH_2PO_4
4.00 g	NaCl
1.08 g	$Na_2HPO_4 \cdot 7H_2O$

Add dd H_2O to 200 mL. Autoclave. Store at room temperature.

Dilution/Wash Buffer (GFP-Trap)

10 mM	Tris-HCl, pH 7.5
150 mM	NaCl
0.5 mM	EDTA

Lysis Buffer (GFP-Trap)

10 mM	Tris-HCl, pH 7.5
150 mM	NaCl
0.5 mM	EDTA
0.5%	NP-40

Store at 4 degrees C. Add appropriate volume of Halt 100x Protease Inhibitor Cocktail (Thermo Scientific) before use.

2x SDS-PAGE Sample Buffer

250 mM	Tris-HCl, pH 6.8
1-%	Glycerol
2%	SDS
0.01%	Bromophenol blue
20 mM	DTT

Add DTT immediately before use.